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Relationship between an Altered Membrane Form and a Low Affinity Form of the β-Adrenergic Receptor Occurring during Catecholamine-induced Desensitization

EVIDENCE FOR RECEPTOR INTERNALIZATION*

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We have investigated the relationship between the catecholamine-induced occurrence in 1321N1 human astrocytoma cells of β-adrenergic receptors that exhibit low apparent affinity for hydrophilic ligands in short-time assays with intact cells and a population of β-adrenergic receptors that migrate in a light vesicle fraction on sucrose density gradients. Pretreatment of cells with concanavalin A prevents the generation of both of these forms of the receptor during incubation with agonists but does not prevent the agonist-induced decrease in isoproterenol-stimulated cyclic AMP production that also occurs during desensitization. Selective labeling of the low affinity β-receptors with ^{125}I-pindolol followed by centrifugation on sucrose density gradients revealed that all of the receptors in the light vesicle fraction from desensitized cells were of the low affinity type, but that a portion of the low affinity receptors also migrated in a heavier sucrose fraction together with the plasma membrane. In contrast, in control cells, no low affinity receptors were present in the heavy sucrose fractions. The agonist-induced occurrence of these various forms of the β-adrenergic receptor can be explained on the basis of current models of desensitization involving agonist-induced internalization of β-adrenergic receptors.

High affinity binding of agonists to β-adrenergic receptors on intact 1321N1 human astrocytoma cells can be demonstrated in short-time (15 s) radioligand-assays (1). During further (>1 min) incubation of cells in the presence of agonist, approximately half of the cellular β-adrenergic receptors are converted from this high affinity form to a form that exhibits low apparent affinity for the agonists isoproterenol and epinephrine (1, 2). This low apparent affinity results, at least in part, from slow attainment of equilibrium binding of these ligands with β-adrenergic receptors (2). The low apparent affinity (slow equilibration) in 1321N1 cells appears to be related to the hydrophilicity of these agonists rather than to the fact that they are agonists, since similar changes in binding are observed for the hydrophilic antagonist sotalol but not for the more lipophilic antagonists metoprolol and propranolol (2). High affinity binding of agonists and agonist-induced conversion of this high affinity form to a form exhibiting much lower affinity has been demonstrated by a number of other laboratories using other cell lines (3–5).

Incubation of cells with agonists also leads to a change in the sedimentation properties of β-adrenergic receptors measured by differential centrifugation (6, 7) or by sucrose density gradient centrifugation (8, 9). In native 1321N1 astrocytoma cells, nearly all of the β-adrenergic receptors migrate in a fraction that contains the enzyme adenylate cyclase and other markers for the plasma membrane (8, 9). Following incubation with agonist, about half of the cellular β-adrenergic receptors are converted to a form that is recovered in a light vesicle fraction at much lower sucrose densities than that of the plasma membrane fraction (8, 9). In analogy with polypeptide hormone receptor systems (10, 11), it has been postulated (8, 9) that this change in sedimentation properties may result from internalization of β-adrenergic receptors within endocytotic vesicles.

In a previous report (2), we postulated that the low apparent affinity and slow equilibration of hydrophilic ligands with the altered β-adrenergic receptors present in cells preincubated with agonists might be explained by receptor internalization, and consequently, reduced accessibility of these receptors to ligands that cross cell membranes slowly. In the experiments reported here, we have directly examined the relationship between the conversion of β-adrenergic receptors to the low apparent affinity form and the appearance of β-adrenergic receptors in the light vesicle fraction. The results indicate that the two phenomena are closely related and support the concept of agonist-induced internalization of β-adrenergic receptors.

EXPERIMENTAL PROCEDURES

Materials—(−)-Isoproterenol bitartrate and (±)-propranolol were obtained from Sigma Chemical Company, ConA[1] was from Calbiochem, and ultrapure sucrose was from Schwarz-Mann. [^{3}H]Adenine and sodium [^{125}I]iodide were obtained from ICN and Amersham, respectively. (−)-Pindolol was a gift from Sandoz Pharmaceuticals, and both nonradioactive and ^{3}H-labeled CGP-12177 were gifts from Dr. M. Staehelin (Ciba-Geigy). ^{125}I-Pindolol was prepared by a modification (12) of the method of Barovsky and Brooker (13).

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[1] The abbreviations are: ConA, concanavalin A; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Eagle's-Hepes, serum-free Eagle's medium containing 20 mM Hepes, pH 7.4; βAR_{LV} and βAR_{LA}, light vesicle and low affinity forms of β-adrenergic receptors.

Cell Culture—Human astrocytoma cells (1321N1) were grown as previously described (1). Cells taken from confluent flasks were seeded in either 35-mm (Falcon) or 150-mm tissue culture dishes at a density of approximately 8000 cells/cm^2 and used on the 4th day following subculture. For desensitization, cells were incubated for 20 min (unless otherwise specified) at 37 °C in growth medium containing 1 μM isoproterenol plus 1 mM sodium ascorbate, and control cells were incubated for 20 min in growth medium with ascorbate alone. Cells were then washed two to three times with Eagle's-Hepes at 37 °C prior to assay.

Pretreatment of Cells with ConA—The medium was aspirated from cell sheets on 35-mm dishes and replaced with Eagle's-Hepes with or without 0.25 mg/ml ConA. The cells were incubated at 37 °C for 30 min. The cells were then incubated an additional 20 min with 1 μM isoproterenol in 1 mM sodium ascorbate (desensitized) or with ascorbate alone (control) in the continued presence or absence of ConA. The cell sheets were rinsed two to three times with Eagle's-Hepes at 37 °C prior to assay.

Competition Binding Assays—Binding to intact cell β-adrenergic receptors in the absence or presence of varying concentrations of isoproterenol or CGP-12177 was measured in 15-s assays with 100 pM ^{125}I-pindolol as previously described (1, 2). Specific binding in the absence of competing ligand was approximately 1500 cpm bound/assay. Nonspecific binding, measured in each experiment as that occurring in the presence of 1 μM propranolol, was about 15% of total binding.

Competition curves were analyzed by computerized nonlinear least-squares curve fitting of the raw data to models for law of mass action interaction of the competing ligand with a single site or with two independent sites as described previously (1, 2). Data are expressed as the percentage of ^{125}I-pindolol specifically bound in the absence of competitor. Standard errors of all points on the curves were generally less than 5% of the amount specifically bound in the absence of competitor. It should be noted that these short-time binding assays provide an estimate of the true affinity for the receptor only for competing ligands that attain equilibrium with the receptor very rapidly (1, 14).

Cyclic AMP Accumulation in Intact Cells—Washed cells were incubated for 1 min at 37 °C in Eagle's-Hepes containing [^{3}H]adenine (5 μCi/ml) and either 10 μM isoproterenol in 1 mM ascorbate or ascorbate alone. This medium was then aspirated and 1 ml of 5% trichloroacetic acid was added. [^{3}H]cAMP was measured as previously described (2).

Prelabeling of Cells and Sucrose Density Gradient Centrifugation—Cells grown on 150-mm dishes were incubated for 2, 5, or 20 min in growth medium with 1 μM isoproterenol plus ascorbate (desensitized) or ascorbate alone (control) and then rapidly washed three times with 10 ml of Eagle's-Hepes at 37 °C prior to labeling. Cells were then incubated for 15 s with 10 ml of 100 pM ^{125}I-pindolol in the absence or presence of various competing ligands. This medium was aspirated and the cells were washed three times at 37 °C with 10 ml of Eagle's-Hepes containing 100 μM propranolol. The dishes were then treated on ice for 5 min with 10 ml of ice-cold Eagle's-Hepes containing 0.25 mg/ml of ConA. This was followed by rinsing with 10 ml of ice-cold lysis buffer (1 mM Tris, pH 7.4, 2 mM EDTA) and a 20-min incubation with lysis buffer on ice. The lysis buffer was aspirated and the cells were lysed by scraping with a rubber policeman.

Sucrose density gradients were formed using an ISCO model 570 gradient former at 4 °C. Gradients consisted of a 1.2-ml cushion of 5% sucrose on top of a 9-ml linear gradient from 30 to 60% sucrose. The lysates from three dishes were combined (2–3 ml) and applied to each gradient. Centrifugation was for 60 min at 35,000 rpm (217,000 × g_{max}) in a Beckman SW 40 Ti rotor and a Beckman L8-70 refrigerated ultracentrifuge at 4 °C. Fractions of 0.6 ml were collected from the top of each gradient with an ISCO model 568 fractionator. The amount of ^{125}I-pindolol in each fraction was then determined in a γ counter. All of the ^{125}I-pindolol recovered from the gradient was apparently β-adrenergic receptor-associated radioactivity, since when labeling was carried out in the presence of 1 μM propranolol, radioactivity was only recovered in the supernatant fractions.

In one experiment, treatment of cells, centrifugation, and fractionation were performed as above, except that the labeling was on ice for 6 h with [^{3}H]CGP-12177 and the subsequent washes were with ice-cold Eagle's-Hepes containing 100 μM propranolol. The radioactivity present in each fraction was then determined by liquid scintillation counting.

Treatment of Gradient Fractions with α-Methylmannoside—Plasma membrane fractions from a sucrose density gradient centrifugation as described above were pooled, diluted 5-fold with lysis buffer, and centrifuged for 20 min at 18,000 rpm (37,000 × g) in a Beckman JA-21 rotor and a Beckman J-21 refrigerated centrifuge at 4 °C. The pellet was resuspended in 0.5 M α-methylmannoside in 10 mM Tris, pH 7.4, and incubated for 10 min at 37 °C. The sample was then cooled and centrifuged on a second sucrose density gradient in a manner identical to the first centrifugation. This gradient was fractionated and the amount of ^{125}I-pindolol in each fraction was determined.

RESULTS

Effects of ConA Pretreatment—Preincubation of 1321N1 cells with ConA has been shown to block the agonist-induced shift of receptors from the plasma membrane to light vesicle fractions (9). However, the agonist-induced decrease in isoproterenol-stimulated adenylate cyclase activity as measured in broken cell preparations was not prevented by preincubation of cells with ConA (9). These results indicated that the "uncoupling" of β-adrenergic receptors from stimulation of adenylate cyclase is a separate event from conversion of receptors to the light vesicle form. To determine the relationship between the conversion of receptors to the low affinity form and these other aspects of desensitization, the effect of ConA on β-adrenergic receptors and adenylate cyclase activity in intact cells was studied.[2]

High affinity binding of isoproterenol was observed in short-time assays with control cells pretreated for 30 min with 0.25 mg/ml ConA (Fig. 1). The amount of ^{125}I-pindolol bound in the absence of competing ligand was the same in cells pretreated with ConA as in cells pretreated with buffer alone (Table I); thus, the treatment with ConA did not alter accessibility of the receptor to the antagonist ^{125}I-pindolol. Pretreatment with ConA also did not alter the binding of isoproterenol to the receptor in control cells as measured in short-time binding assays. Computer analysis of the isoproterenol competition curve from control cells (Fig. 1) indicated that 86 ± 3% of ^{125}I-pindolol binding was inhibited with an IC_{50} of 83 nM and the remaining 14% was inhibited with an IC_{50} of 180 μM; these results are essentially the same as those obtained previously in cells not pretreated with ConA (1, 2).

When cells that had not been treated with ConA were preincubated for 20 min with isoproterenol, approximately 50% of the β-adrenergic receptors were converted from the high affinity form to the low affinity form (Fig. 1, Table I, and Ref. 2). In contrast, when cells pretreated with ConA were exposed to isoproterenol, there was only a slight increase in β-adrenergic receptors in the low affinity form (Fig. 1 and Table I). In addition, there appeared to be an approximately 2-fold increase in the IC_{50} at the high affinity site. Computer analysis of the curve for isoproterenol-pretreated cells in Fig. 1 indicated that 76% of ^{125}I-pindolol binding was inhibited with an IC_{50} of 160 ± 35 nM and 24% with an IC_{50} of 730 μM.

[2] This use of ConA should be distinguished from the routine use of ConA in experiments involving sucrose density gradient centrifugation. In the experiment depicted in Fig. 1 and Tables I and II, ConA pretreatment was carried out *prior* to the induction of desensitization by isoproterenol at 37 °C. This use of ConA is based on our previous report demonstrating that ConA blocks the catecholamine-induced change in the membrane form of β-adrenergic receptors (9). However, in all experiments involving sucrose density gradient centrifugation, *e.g.* Figs. 2, 4, 5, 6, and 7, ConA treatment of the cells was carried out on ice subsequent to any incubation of the cells with isoproterenol, CGP-12177, and/or ^{125}I-pindolol and just prior to hypotonic lysis of the cells and sucrose density gradient centrifugation. This use is based on the more effective separation of light vesicle and plasma membrane β-adrenergic receptors following ConA treatment (8, 9, 20).

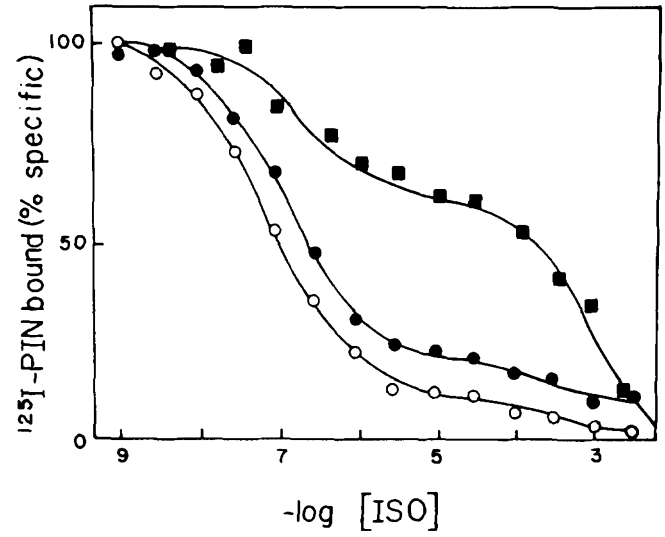

FIG. 1. **Effect of ConA on the isoproterenol-induced formation of a low affinity form of β-adrenergic receptors.** Cells were incubated for 30 min in the absence (■) or presence (○, ●) of 0.25 mg/ml ConA, and then for an additional 20 min with 1 μM isoproterenol (*ISO*) plus ascorbate (■), isoproterenol plus ascorbate and 0.25 mg/ml ConA (●), or ascorbate alone and 0.25 mg/ml ConA (○). The cells were washed and binding of ^{125}I-pindolol (*PIN*) was determined in the presence of the indicated concentrations of isoproterenol in 15-s assays as described under "Experimental Procedures." The experimental points shown are the average of three experiments performed in duplicate.

TABLE I

Effects of pretreatment of cells with ConA on ligand binding to β-adrenergic receptors

1321N1 cells were pretreated (30 min) in the absence or presence of 0.25 mg/ml ConA and then in the absence or presence (20 min) of 1 μM isoproterenol as described under "Experimental Procedures." The cells were then washed and incubated with 100 pM ^{125}I-pindolol for 15 s in the absence or presence of 10 μM isoproterenol.

30-min incubation	20-min incubation	^{125}I-Pindolol specifically bound		Low apparent affinity
		Presence of 10 μM isoproterenol	Absence of isoproterenol	
		cpm		%
−ConA	−Isoproterenol	252	1614	16
−ConA	+Isoproterenol	969	1615	60
+ConA	−Isoproterenol	248	1626	15
+ConA	+Isoproterenol	352	1568	23

TABLE II

Effects of pretreatment of cells with ConA on cyclic AMP accumulation

1321N1 cells were treated with ConA and isoproterenol as described in Fig. 1. The cells were then washed and [^{3}H]cAMP accumulation was measured as described under "Experimental Procedures."

30-min incubation	20-min incubation	[^{3}H]cAMP formed		Decrease during desensitization
		Basal	+10 μM Isoproterenol	
		cpm		%
−ConA	−ISO	189	2130	
−ConA	+ISO	231	954	55
+ConA	−ISO	187	1911	
+ConA	+ISO	282	1059	45

Although ConA almost completely blocked the agonist-induced conversion of receptors to the low affinity form, it had only a minimal effect on the agonist-induced loss of isoproterenol-stimulated cyclic AMP accumulation. A 20-min exposure to agonist caused a comparable decrease in isoproterenol-stimulated cyclic AMP accumulation in both control and ConA-pretreated cells (Table II). Pretreatment with ConA did not cause a change in the basal level of cyclic AMP accumulation in control or desensitized cells, but did cause a small decrease in the level of isoproterenol-stimulated cyclic AMP accumulation in control cells. However, the major effect of ConA apparently is not on the interaction of isoproterenol with β-adrenergic receptors or on the coupling of β-adrenergic receptors to adenylate cyclase but rather is on the subsequent conversion of the receptors to the low affinity form.

Distribution of Low Affinity Receptors on Sucrose Density Gradients—The relationship between β-adrenergic receptors exhibiting high and low apparent affinity for isoproterenol in short-time assays with intact cells and those migrating in the plasma membrane and light vesicle fractions on sucrose density gradients was assessed directly. Control and desensitized cells were labeled with ^{125}I-pindolol for 15 s in the absence or presence of 1 μM propranolol or 10 μM isoproterenol. Propranolol at 1 μM blocks binding of ^{125}I-pindolol to all β-adrenergic receptors, whereas 10 μM isoproterenol selectively blocks binding of ^{125}I-pindolol to the high affinity receptors without blocking binding to the low affinity receptors (see Fig. 1 and Ref. 2). The cells were then washed at 37 °C to remove ^{125}I-pindolol not bound to β-adrenergic receptors, treated with ConA, lysed, and subjected to sucrose density gradient centrifugation. The small amount of ^{125}I-pindolol bound in the presence of 1 μM propranolol (nonspecific binding) did not enter the sucrose gradient and, thus, did not interfere with quantitation of labeling of receptors in the light vesicle and plasma membrane fractions.

When control cells were labeled in the absence of isoproterenol, the bulk of bound ^{125}I-pindolol migrated in the plasma membrane fraction with only a small amount migrating in the light vesicle fraction (Fig. 2*B*). The presence of 10 μM isoproterenol during the ^{125}I-pindolol-labeling step essentially completely blocked labeling of receptors in the plasma membrane fraction; some labeling of the light vesicle fraction still occurred (Fig. 2*B*). Thus, in control cells, all of the labeled β-adrenergic receptors in the plasma membrane fraction exhibit high affinity for isoproterenol, whereas a population of receptors exhibiting low affinity for isoproterenol can be identified in the light vesicle fraction.

When desensitized cells were labeled in the absence of isoproterenol, bound ^{125}I-pindolol was distributed approximately equally between the plasma membrane and light vesicle fractions (Fig. 2*A*). These results, obtained by labeling of β-adrenergic receptors on intact cells prior to gradient centrifugation, are similar to those obtained in previous studies (8, 9) employing membrane-binding assays of β-adrenergic receptor distribution after gradient centrifugation. The presence of 10 μM isoproterenol during the ^{125}I-pindolol labeling did not decrease binding to β-adrenergic receptors in the light vesicle fraction; indeed, in some experiments, the amount of labeling actually increased (Fig. 2*A*). In contrast to the complete inhibition of labeling of the plasma membrane receptors in control cells, ^{125}I-pindolol binding to β-adrenergic receptors in the plasma membrane fraction of desensitized cells was only partially blocked by 10 μM isoproterenol. Thus, in desensitized cells, all of the receptors in the light vesicle fraction exhibit low apparent affinity for isoproterenol, whereas the plasma membrane fraction contained both high and low affinity receptors.

Studies with CGP-12177—The recently developed β-adrenergic receptor ligand CGP-12177 is an antagonist that has

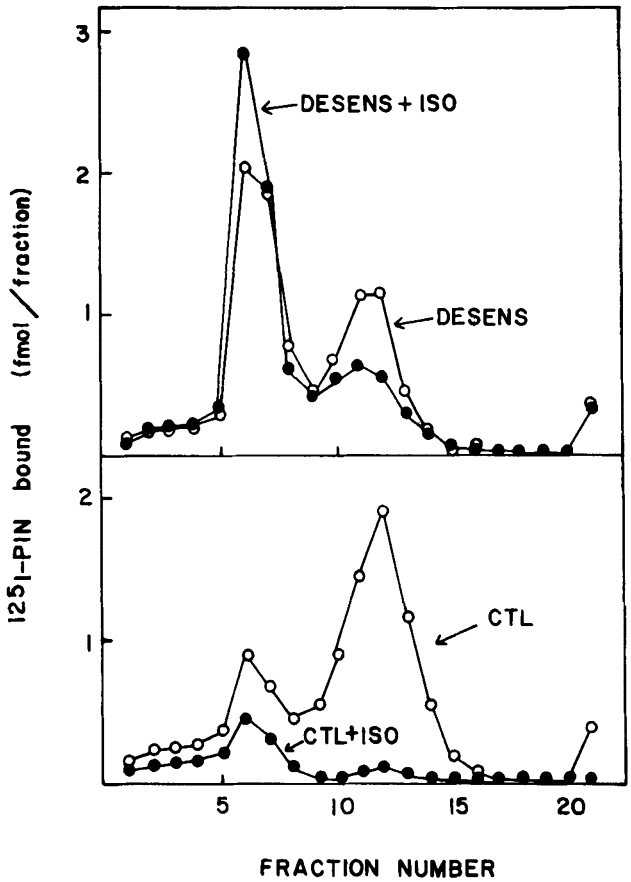

FIG. 2. **Selective labeling of affinity forms of the β-adrenergic receptor revealed by sucrose density gradient centrifugation.** Control (*CTL*) and desensitized (*DESENS*) cells were washed and labeled for 15 s with ^{125}I-pindolol (*PIN*) in the absence (○) or presence (●) of 10 μM isoproterenol (*ISO*). After washing to remove ^{125}I-pindolol not bound to β-adrenergic receptors, the cells were treated with ConA and lysed, and the lysates were centrifuged on sucrose density gradients as described under "Experimental Procedures." The results shown are from a single experiment; similar results were obtained in four additional experiments.

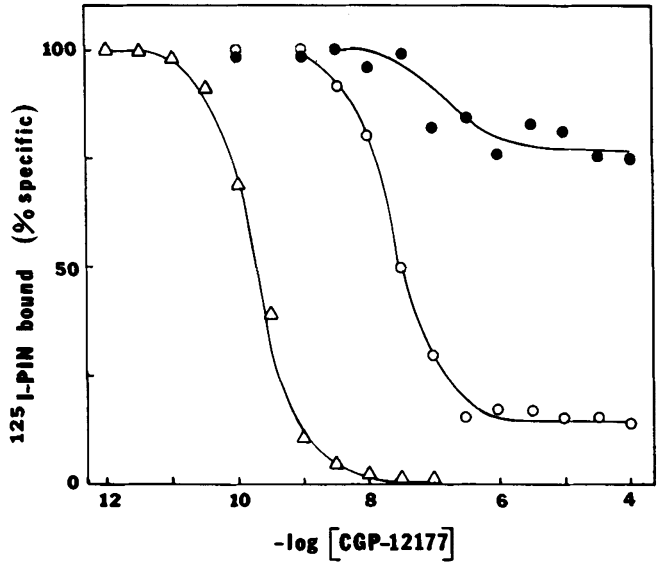

FIG. 3. **Effect of CGP-12177 on the binding of ^{125}I-pindolol to intact control or desensitized 1321N1 cells.** Control (○) or desensitized (20 min; ●) cells were incubated for 15 s (○, ●) in the presence of 100 pM ^{125}I-pindolol (*PIN*) and the indicated concentrations of CGP-12177. Alternatively, naive cells were incubated for 60 min in the presence of the indicated concentrations of CGP-12177 prior to performing 15-s assays of ^{125}I-pindolol binding in the presence of the indicated concentrations of CGP-12177 (△). The *experimental points* shown are from one (△) or two (○, ●) experiments performed in duplicate.

been reported to bind only to receptors on the surface of intact cells (15). This ligand, like isoproterenol, epinephrine, and sotalol (2), competed with high apparent affinity for only about 84% of the ^{125}I-pindolol-binding sites in short-time assays with control cells (Fig. 3). In desensitized cells, only about 27% of the ^{125}I-pindolol binding was inhibited with high affinity by CGP-12177 (Fig. 3). Further inhibition of ^{125}I-pindolol binding under these short-time assay conditions was not seen at concentrations of CGP-12177 as high as 100 μM in either control or desensitized cells. It should be noted that the apparent affinity of β-adrenergic receptors for CGP-12177 under these assay conditions (30–50 nM) is much lower than the true affinity (0.15 nM) measured in equilibrium assays (Fig. 3). The high affinity of this ligand would be expected to result in slow equilibration even with those receptors exposed on the cell surface and thus readily accessible to CGP-12177.

The distribution of high and low affinity binding sites for CGP-12177 between the plasma membrane and light vesicle fractions on sucrose density gradients was determined as described above for isoproterenol, with 10 μM CGP-12177 chosen as a concentration to block high affinity sites without altering binding to low affinity sites. The results obtained (Fig. 4) were completely analogous to those obtained with 10 μM isoproterenol (Fig. 2). In control cells, 10 μM CGP-12177 blocked labeling of receptors in the plasma membrane fraction with minimal effect on labeling of light vesicle receptors. In desensitized cells, 10 μM CGP-12177 did not inhibit labeling of light vesicle receptors, and labeling in the plasma membrane fraction was only partially blocked.

Previous studies in other cell lines have used [^{3}H]CGP-12177 binding on ice to selectively label receptors exhibiting high affinity (rapid accessibility) for this ligand on intact cells. This fraction of β-adrenergic receptors presumably represents cell surface receptors (15–18). When such an experiment was performed with 1321N1 cells (Fig. 5), selective labeling of β-adrenergic receptors in the plasma membrane fraction occurred in both control and desensitized cells. As expected, there were fewer labeled β-adrenergic receptors in the plasma membrane fraction from desensitized cells. No labeling of β-adrenergic receptors in the light vesicle fraction of control or desensitized cells was detected.

Evidence against Lysosomal β-Adrenergic Receptors—The occurrence of receptors exhibiting low apparent affinity for isoproterenol and CGP-12177 in the plasma membrane fraction was a somewhat unexpected result based on our hypothesis that the basis of such low affinity is sequestration of receptors within internalized vesicles. Therefore, the nature of these receptors was investigated further. Since secondary lysosomes are known to migrate at a sucrose density similar to that of the plasma membrane (10, 11, 19, 20), we explored the possibility that the low affinity receptors occurring in the plasma membrane fraction might be receptors that had been transferred from endocytotic vesicles to lysosomes. Since the transfer of receptors to lysosomes typically requires 10–20 min (10, 11, 19), the proportion of receptors labeled in the presence of 10 μM isoproterenol and migrating in the plasma membrane fraction would be expected to increase with increasing times of desensitization. In fact, the ratio of "plasma membrane" to light vesicle labeling in the presence of isoproterenol was essentially constant for cells pre-exposed to iso-

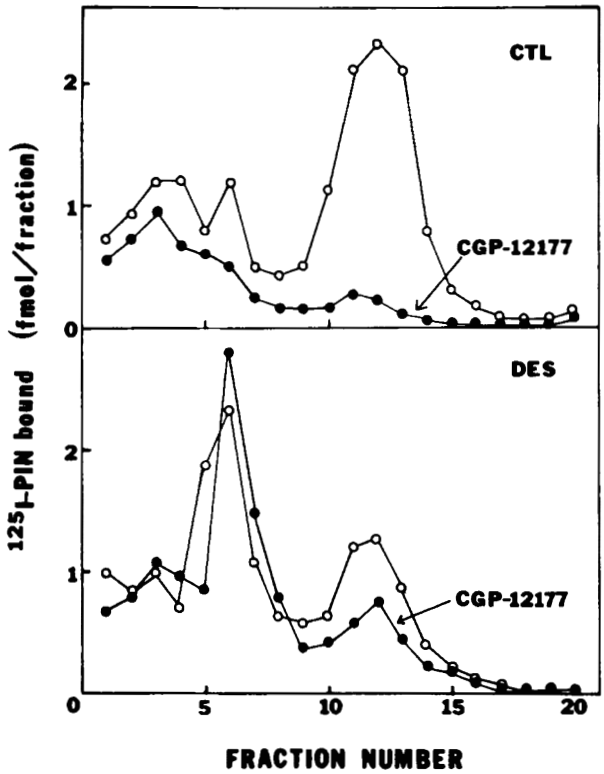

FIG. 4. **Selective inhibition by CGP-12177 of labeling of different membrane forms of β-adrenergic receptors.** Control (*CTL*) and desensitized (*DES*) cells were washed and labeled for 15 s with ^{125}I-pindolol (*PIN*) in the absence (○) or presence (●) of 10 μM CGP-12177. After washing to remove ^{125}I-pindolol not bound to β-adrenergic receptors, the cells were treated with ConA and lysed, and the lysates were centrifuged on sucrose density gradients as described under "Experimental Procedures." The results shown are from a single experiment; similar results were obtained in one additional experiment.

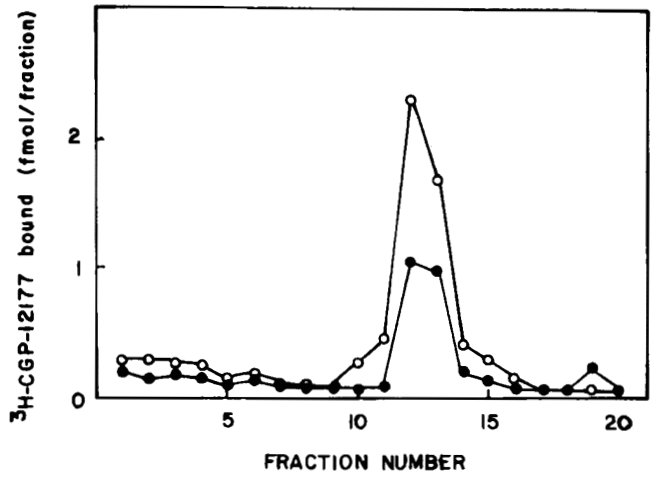

FIG. 5. **Selective labeling of plasma membrane β-adrenergic receptors with [^{3}H]CGP-12177.** Control (○) and desensitized (●) cells were washed and incubated with 1 nM [^{3}H]CGP-12177 for 6 h on ice. After washing to remove CGP-12177 not bound to β-adrenergic receptors, the cells were treated with ConA and lysed, and the lysates were centrifuged on sucrose density gradients as described under "Experimental Procedures." The results shown are from a single experiment.

proterenol for 2, 5, or 20 min (Fig. 6). This result argues against a transfer of endocytotic vesicles to lysosomes as an explanation for this phenomenon.

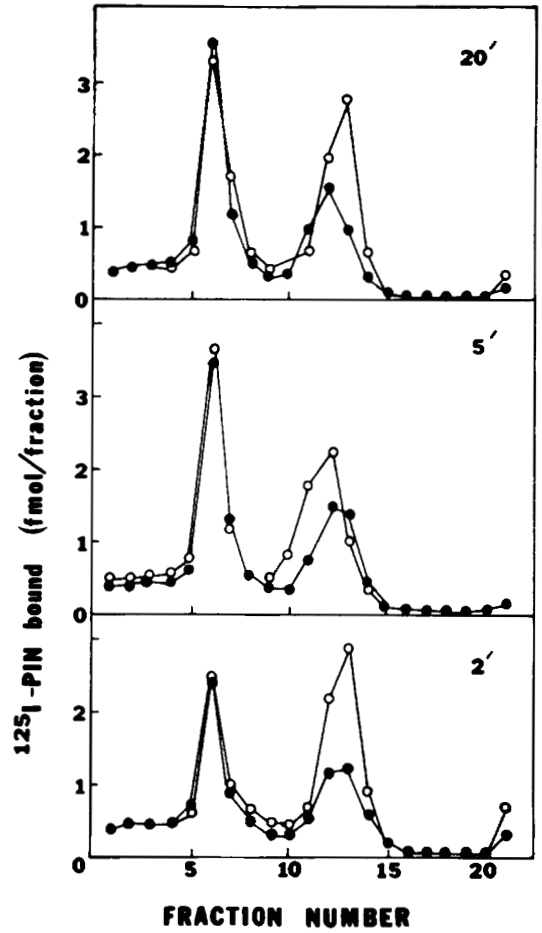

FIG. 6. **Time course of appearance of β-adrenergic receptors in different affinity forms assessed by sucrose density gradient centrifugation.** Cells were incubated for 2, 5, or 20 min in growth medium containing isoproterenol plus ascorbate, washed, and then labeled for 15 s with ^{125}I-pindolol (*PIN*) in the absence (○) or presence (●) of 10 μM isoproterenol. After washing, labeled cells were treated with ConA and lysed, and the lysates were centrifuged on sucrose density gradients. The results shown are from a single experiment.

Another test for association of these receptors with lysosomes was to treat isolated fractions with α-methylmannoside to dissociate bound ConA and then centrifuge the sample on a second sucrose density gradient. This treatment results in a shift of plasma membrane markers to a lighter sucrose density (20), whereas lysosomal markers continue to sediment at high sucrose densities (9, 20). When such an experiment was performed with plasma membrane fractions labeled in the presence of 10 μM isoproterenol (Fig. 7), the majority of the bound ^{125}I-pindolol was found in a lighter density fraction, suggesting that most of these receptors are associated with the plasma membrane and not with lysosomes. A significant amount of bound ^{125}I-pindolol would be expected to dissociate from the receptor during the α-methylmannoside treatment at 37 °C; this is the most likely explanation for the low recovery of bound ^{125}I-pindolol and for the higher level of radioactivity in the first five (soluble) fractions of the second gradient.

DISCUSSION

Incubation of 1321N1 human astrocytoma cells in the presence of β-adrenergic receptor agonists leads to several changes in the β-adrenergic receptors and in coupling to adenylate

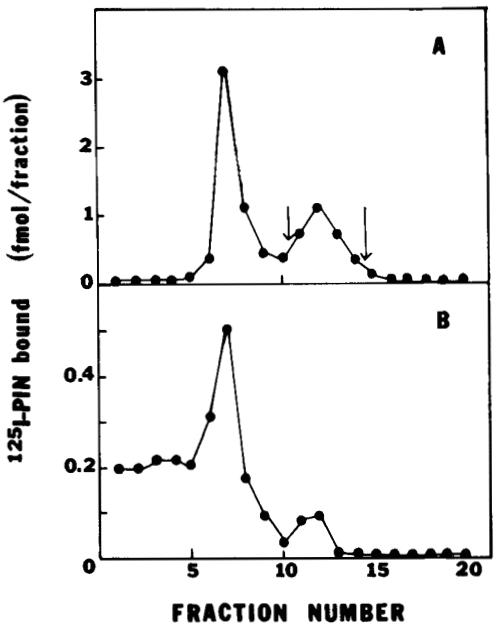

FIG. 7. **Effect of α-methylmannoside on the sucrose density gradient distribution of β-adrenergic receptors.** Desensitized cells were washed and then labeled for 15 s with ^{125}I-pindolol (*PIN*) in the presence of 10 μM isoproterenol. The amount of ^{125}I-pindolol bound in a 100-μl aliquot of each fraction was determined (first gradient). The remainder of the plasma membrane fractions (11–14) were pooled and treated with α-methylmannoside as described under "Experimental Procedures." This sample was then centrifuged on a second sucrose density gradient. The results shown are from a single experiment; similar results were obtained in a second experiment using a slightly different protocol.

cyclase; these events are collectively referred to as desensitization (21). Two of these changes, the conversion of receptors to a light vesicle form identified on sucrose density gradients (βAR_{LV}) and the conversion of receptors from a form exhibiting high affinity for various hydrophilic ligands to a form exhibiting low apparent affinity in short-time assays with intact cells (βAR_{LA}), have been postulated to result from internalization of βAR within endocytotic vesicles (2–8, 9). The experiments described here were designed to investigate the relationship of these two aspects of desensitization and to further define the nature of the form(s) of the receptor exhibiting these properties.

Previous studies had shown that the time courses for these two phenomena were similar; both occur after a lag of 30–60 s and then proceed with a half-time of 1–2 min (2, 9). Both of these phenomena involve conversion of approximately half of the cellular receptors to the altered forms, and both are reversible with similar time courses (2, 9). In addition, Fig. 1 shows that the formation of βAR_{LA} during exposure to isoproterenol, like the formation of βAR_{LV} (9), is prevented if cells are pretreated with ConA. However, in agreement with results obtained previously using assays of adenylate cyclase activity in membrane preparations (9), the uncoupling of the stimulation of cAMP formation by isoproterenol is essentially unaltered by treatment with ConA. These results indicate that βAR_{LV} and βAR_{LA} are formed by a common process and are in fact different reflections of a single form of the receptor. The results with ConA (Table II) also support our previous proposal (9) that uncoupling of β-adrenergic receptors from stimulation of adenylate cyclase is a separate reaction that occurs prior to the formation of βAR_{LV}.

More direct evidence in favor of this relationship between light vesicle receptors and low affinity receptors comes from the experiment shown in Fig. 3. The receptors that migrate in the light vesicle fraction in desensitized cells clearly exhibit low affinity for isoproterenol in short-time assays with intact cells; this follows since they were labeled with ^{125}I-pindolol in the presence of a concentration of isoproterenol that blocks binding to high affinity but not to low affinity sites. Although the results are less clear, it also appears that the small number of receptors found in the light vesicle fraction from control cells includes the small population of receptors that exhibits low affinity for isoproterenol in short-time assays with control cells (Figs. 1 and 3 and Ref. 2). Thus, results from both control and desensitized cells support the identity of βAR_{LV} and βAR_{LA}.

While all of the βAR_{LV} from desensitized cells appeared to exhibit low affinity for isoproterenol, some low affinity receptors also were found in the plasma membrane fraction. Experiments designed to test the hypothesis that these receptors were associated with lysosomes (Figs. 6 and 7) proved negative. The shift of these labeled receptors to a lighter sucrose density following treatment with α-methylmannoside provides strong evidence that these receptors are associated with the plasma membrane. If in fact the low apparent affinity results from receptor internalization and therefore limited accessibility to isoproterenol as we postulate, then the low affinity receptors associated with the plasma membrane may represent an intermediate step in the internalization pathway. The nature of this subpopulation of receptors is under further investigation.

The recently developed β-adrenergic receptor antagonist CGP-12177 is a hydrophilic ligand that has been used in several other experimental systems to provide evidence for receptor internalization (15–18). In short-time assays (Fig. 3), this ligand distinguishes two populations of β-adrenergic receptors on intact 1321N1 cells that occur in similar proportions to those detected by isoproterenol, epinephrine, and sotalol (2). The apparent inability of CGP-12177, even at high concentrations, to inhibit labeling of a small fraction of the receptors in control cells results from limited accessibility to those receptors and therefore slow equilibration. Thus, when equilibrium binding was allowed to occur with various concentrations of CGP-12177 prior to exposure to ^{125}I-pindolol (Fig. 3), all of the ^{125}I-pindolol-binding sites exhibited high affinity for CGP-12177. Presumably, the same phenomenon is responsible for the inability of CGP-12177 to block ^{125}I-pindolol binding in short-time assays to the much larger fraction of these receptors (βAR_{LA}) in desensitized cells. These results are thus similar to those previously described with isoproterenol and sotalol (2). In addition, the distribution of high and low affinity sites for CGP-12177 between the plasma membrane and light vesicle fractions is essentially identical to that for isoproterenol (Fig. 4), suggesting that these two ligands are differentiating the same forms of the receptor.

The use of nonradioactive CGP-12177 or other hydrophilic antagonists in competition for ^{125}I-pindolol binding to study the various forms of the receptor is preferable to the use of [^{3}H]CGP-12177 (17, 18) for several reasons. First, the use of ^{125}I-pindolol as the radioligand allows labeling of all receptors and competition with CGP-12177 allows the distinction, by selective labeling, of low affinity receptors; thus, the distribution of both high and low affinity receptors can be determined using the same radioligand (Fig. 4). In contrast, the use of [^{3}H]CGP-12177 gives no information about the distribution of the low affinity receptors (Fig. 5 and Refs. 17 and 18). Secondly, the higher specific activity of ^{125}I-pindolol makes possible studies at short times of assay, which is critical for studying desensitized receptors in intact cells. Further-

more, ^{125}I-pindolol makes feasible studies in cells containing relatively few β-adrenergic receptors such as the 1321N1 cells studied here.

The results presented here provide strong evidence for a direct relationship between βAR_{LV} and βAR_{LA}. These results, together with previous studies in this (2, 8, 9) and other experimental systems (3–7, 17, 18, 21), also provide strong circumstantial evidence for the localization of these forms of the β-adrenergic receptors within endocytotic vesicles. Further studies will no doubt proceed on the assumption that this is the case. However, definitive proof of the intracellular location of these receptors will require direct demonstration using techniques such as electron microscope autoradiography or fluorescence microscopy of β-adrenergic receptors labeled with appropriate markers. These studies await development of more suitable markers for labeling of β-adrenergic receptors on intact cells.

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